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Original article

# Synthesis and *in vitro* activity of novel *N*-3 acylated TSAO-T compounds against HIV-1 and HCV

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ABSTRACT

Preparation of a small library of derivatives of the potent HIV-1 Reverse Transcriptase inhibitor TSAO-T bearing mono or di-carbonyl substituents (designed after docking analysis) at position *N*-3 is reported. A one-pot synthetic methodology has been developed that involves: (i) mono-reaction of TSAO-T with glutaryl dichloride under phase transfer conditions and (ii) *in situ* acyclic substitution of the remaining chloro atom by oxygen or nitrogen nucleophiles. The method is compatible with the polyfunctionality of the TSAO-T molecule, proceeds with high conversion yields and allows introducing molecular diversity. The anti-HIV-1 and -HCV activity was studied in cell culture. The new *N*-3 acylated TSAO-T derivatives are active against HIV-1 (nanomolar range). Anti-HCV activity was observed in the micromolar range, that is at compound concentrations that were found cytostatic against human T-lymphocytes.



## 1. Introduction

[2′,5′-Bis-*O*-(*tert*-butyldimethylsilyl)-β-D-ribofuranosyl]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) thymine (TSAO-T) [1,2] represents the prototype of a unique class of nucleoside analogues with human immunodeficiency virus type-1 (HIV-1) Reverse Transcriptase (RT) inhibitory activity, acting through a non competitive mechanism of enzyme inhibition [3–5]. Resistance of HIV-1 to TSAO-T type compounds has been observed by selection of the E138K mutation in the virus-encoded RT [6]. The phenomenon of resistance and the interest in the complete elucidation of the precise binding mode of this family of compounds to HIV-1 RT has strongly stimulated the exploration of structural modifications of TSAO-T analogues.

Introduction of functionnal groups at the *N*-3 position has been studied by Camarasa and colleagues. Thus, a variety of polar, lipophilic, or aromatic groups linked to the *N*-3 position through flexible polymethylene linkers of different length [7,8] or unflexible unsaturated bonds [7,9] have been synthesised. In order to form covalent bonds between the novel TSAO-T derivatives and the HIV-1 RT amino acids in the NNRTI binding pocket, series of compounds which incorporate a photoreactive moiety, a strong electrophilic functional group [7,9] or amino acids [10] on this nitrogen have also been described. Among all the *N*-3 modifications carried out on TSAO-T derivatives, preparation of the *N*-methylcarbamoyl derivative **1** was the more efficient as it turned out to be 6-fold more effective in comparison with TSAO-T ($EC_{50}$ = 0.01 μM). This gain of activity has been explained after docking studies by the formation of two hydrogen bonds with the enzyme, one with Pro-B140 and one with Lys-B49. Previous research carried out in our group indicated that acylation at the *N*-3 position of TSAO-T was also a valid strategy in the search of new and more active HIV-1 RT inhibitors. Thus, *N*-3-Boc derivative **2** turned to be 3-fold more potent than TSAO-T as a result from a presumed additional hydrogen bond between the Boc carbonyl oxygen and Lys-A172 [11–13]. So a carbonyl group at the *N*-3 position of the base seems to be favourable for establishing new binding interactions with the enzyme. Docking studies suggested that associated bulky substituents restrict the proper adjustment of the ligand inside the binding site. On the contrary, we may speculate that compounds bearing an unhindered

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carbonyl group on this position could improve activity or resistance profiles by optimizing the H-bond interaction and reducing the unfavourable steric hindrance. Thus, we hypothesize that the presence of a *N*-3 acylated not-hindered bulky substituent (compound **A**) or a *N*-3 substituent which combined simultaneously all the functional groups present in compounds **1** and **2** (compound **B**) might provide additional interactions with the enzyme, thus destabilizing the dimer interface and improving inhibitory activity.

On the other hand, the single-stranded RNA flavivirus hepatitis C virus (HCV) affects about 170 million people is a major contributor to cirrhosis and hepatocellular carcinoma (HCC), and is one of the most common indications for liver transplantation [14]. HCV and HIV share routes of transmission, and thus, the prevalence of HCV infection is 15–30% among HIV-infected patients [15]. In HIV-positive patients, longer survival may permit the progression of HCV-related liver disease and increased mortality due to its complications. Some studies have described the faster progression of HCV-related liver disease and higher HCV viral loads in HIV/HCV-coinfected individuals [16]. Thus, discovery of compounds that could be inhibitors of HIV and HCV is a great challenge [17,18]. Therefore, the compounds were first tested against HIV-1 in CEM cell cultures, but, in addition, also to HCV in human Huh-7 cell cultures.

The HCV polymerase structure shares the same general right-handed configuration consisting of finger, thumb, and palm domains observed in HIV RT. Despite this analogy, four different allosteric binding sites have been discovered in the NS5B polymerase, whereas only one binding site for non-competitive inhibitors has been described for HIV-RT. Thus, a large structural variety of non-competitive inhibitors can be used to potentially interact with NS5B polymerase for HCV [19].

In this context, we report an efficient methodology for direct acylation of TSAO-T, using phase transfer conditions, suitable for library generation of *N*-3 acylated compounds bearing one or two carbonyl groups linked by a C3 alkyl or aryl chain as a model as well as their biological evaluation as anti-HIV inhibitors and also their biological evaluation as potential HCV inhibitors.

## 2. Results and discussion

### 2.1. Computational chemistry approach

In order to rationalize our starting hypothesis presuming additional bindings with derivatives with two carbonyls (Fig. 1, **B**, **C**), and before the recently reported crystal structure of the complex TSAO-T//HIV-1 RT [20], we first performed a docking analysis using compound **B** as a model. Besides the presumedH-bond interactions with Lys-A101, Lys-A103 and Glu-B138, compound **B** may establish four additional hydrogen bonds, as shown in Fig. 2: between the (carbamate) carbonyl oxygen and the Nζ of Lys-A172 (in analogy with the Boc derivatives), between the (carbamate) carboxylic oxygen (not sterically impeded by a bulky neighbour) and the hydroxyl group of Thr-B139, between the (amide) carbonyl and the Nζ of Lys-B49 and, finally, between the amide NH and the hydroxyl group of Thr-A165 instead of Pro-B140.

### 2.2. Synthesis

The reported synthesis of **2** implies incorporation of the Boc substituent in a step prior to the generation of the 4″-amino-oxathiol ring, and is not well-suited for molecular diversity-oriented strategies [11–13]. In this context, we first endeavored in the development of an efficient synthetic methodology for the direct introduction of an acyl substituent at the *N*-3 position of the preformed TSAO-T molecule compatible with the polyfunctionality of the molecule, particularly with the presence of an amino group at C-4″. Attempts to prepare the corresponding 3-*N*-benzoyl derivative **3** (Scheme 1) by reaction with benzoyl chloride using a battery of base catalysts (pyridine, dimethylaminopyridine, triethylamine, sodium hydride, potassium carbonate) were unsuccessful, the starting material being recovered unreacted. Longer reaction times or high temperatures resulted in hydrolysis of the silyl ether groups, known to be essential for TSAO-T biological activity.

The lack of reactivity of the imide nitrogen in TSAO-T towards acylation under the above conditions was probably due to a low

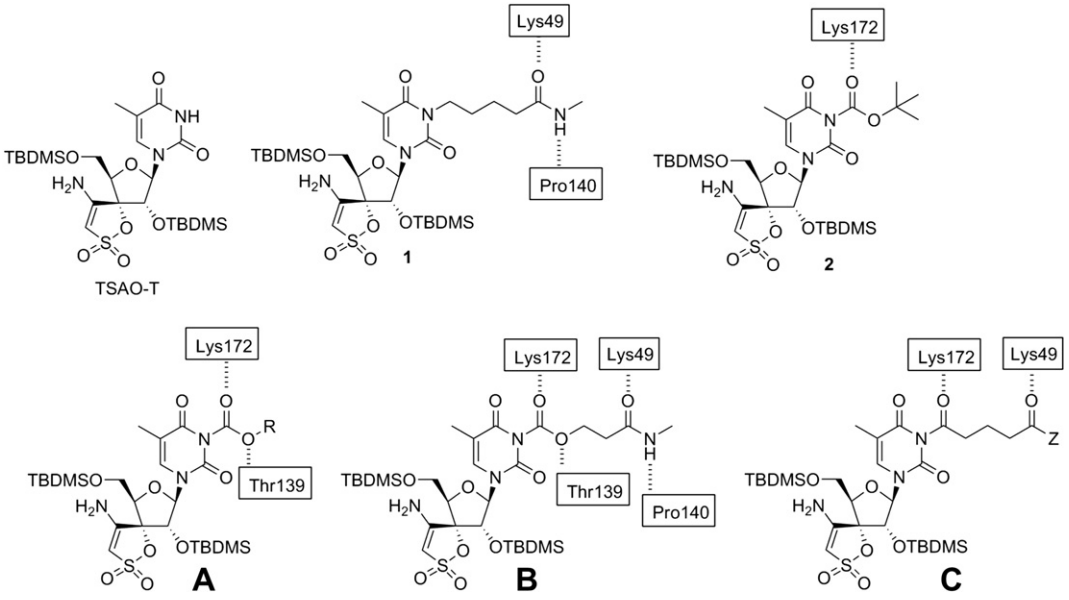



**Fig. 1.** Structures of TSAO-T, *N*-3 substituted analogues **1** and **2**, new derivatives reported in this work (**A** and **C**), and the model compound used for docking analysis (**B**). The suggested interactions of the substituent with amino acids in HIV-1 RT are indicated with hash bonds.

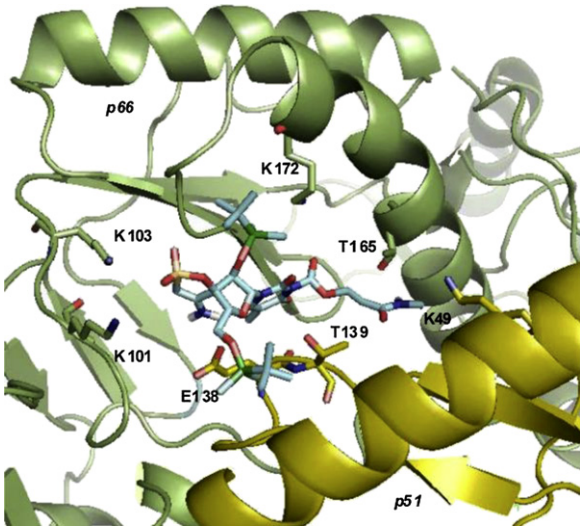
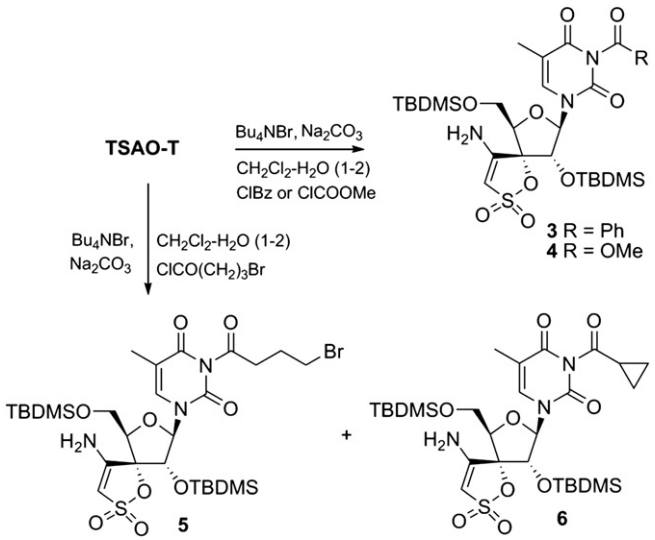


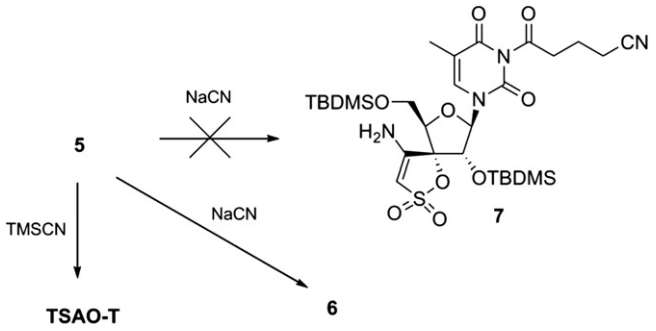

**Fig. 2.** Predicted binding mode for compound **B**. Residues relevant to the discussion are displayed as sticks with carbon atoms colored according to their subunit. Ligand **B** is displayed as sticks with carbon atoms in cyan.

reactivity of the ion pair formed after generation of the corresponding imidure anion. To overcome this problem, acylation with benzoyl chloride (1.2 equivalents)-sodium carbonate (8 equivalents; 0.23 M in water) in dichloromethane-water (1:2), using tetrabutylammonium bromide as phase transfer catalyst, was attempted [21]. The reaction proceeded cleanly at room temperature. The desired benzimide **3** was the sole reaction product when short reaction times (<1 h) were applied. Longer reaction times resulted in concomitant formation of the 3-*N*, 4″-*N* diacylated derivative as a side-product, as detected by mass spectrometry of the crude reaction product. The 3-*N*-methoxycarbonyl TSAO-T derivative **4** was likewise obtained as a closely related analogue of **3** for comparative purposes (Scheme 1).

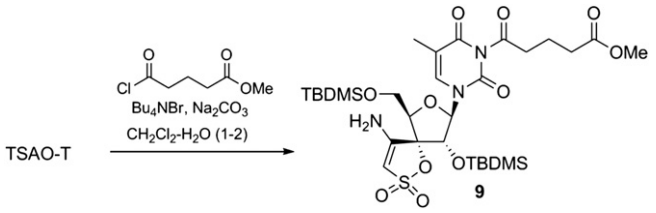
In order to integrate a second carbonyl functionality according to our molecular design (structure **C** in Fig. 1), acylation of TSAO-T with 4-bromobutanoyl chloride using the above optimized phase transfer methodology was next undertaken.




**Scheme 1.** Acylation route using 4-bromobutanoyl chloride.




**Scheme 2.** Cyanation of **5**.

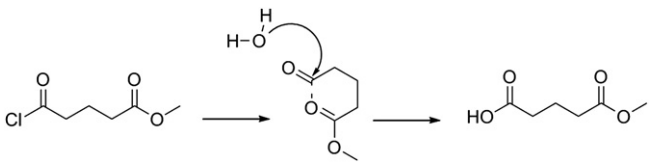



**Scheme 3.** Acylation using glutaric acid route.

In this case the reaction was found to be sensitive to the base concentration. Thus, using sodium carbonate at 0.23 M, a mixture of the desired 4-bromobutanoyl derivative **5** and of the cyclopropylcarbonyl product **6**, arising from **5** by intramolecular cyclization, was obtained (Scheme 1). This side-reaction could be avoided by decreasing the base concentration to 0.11 M. However, attempts to further elaborate compound **5** by replacement of the bromo atom by a nitrile group (compound **7**), a direct precursor of the target compounds, was ineffective. Reaction with sodium cyanide [22] afforded exclusively the cyclopropyl derivative **6**, while the trimethylsilyl cyanide-methanol system provoked hydrolysis of the acyl group to give the starting compound TSAO-T (Scheme 2).

In view of the above-commented difficulties, an alternative convergent approach involving the coupling reaction of TSAO-T with glutaric acid monomethyl ester chloride, as an example of acyl chloride already bearing the desired di-carbonyl motif, was considered (Scheme 3). However, in this case, a fast hydrolysis of the acylating reagent was observed under phase transfer conditions. Nevertheless, formation of di-carbonyl derivative **9** was detected by mass spectrometry when high molar ratios of the acyl chloride were used, though in low yields. Most probably, the carbonyl oxygen of the ester group assists the nucleophilic displacement of the chloro group by water intramolecularly through a six-membered ring intermediate (Scheme 4).

We reasoned that the use of the corresponding diacyl dichloride would slow this side reaction, thereby favouring the intermolecular process. Thus, reaction of TSAO-T with 3.5 equivalents of glutaryl dichloride in phase transfer conditions at 8 °C allowed the formation of the mono-imidation product **8**. *In situ* displacement of the




**Scheme 4.** Nucleophilic displacement of the chloro group by water on 4-chloro glutaric acid monomethyl ester.

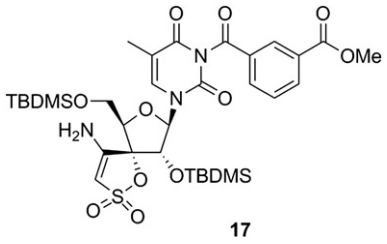



**Fig. 3.** Methyl isophtaloyl ester derivative **17**.

remaining chloro group by nucleophiles like methanol, ammonia, methylamine, dimethylamine sodium methanthiolate, *tert*-butanol, benzylalcohol and benzylamine afforded the corresponding *N*-3 substituted di-carbonyl TSAO-T derivatives **9**–**16** in 36–76% yields after HPLC purification (conversion yields monitored by $^{1}$H NMR were all in the range of 85–95%) (Scheme 5).

The same acylation condition reaction was performed with isophtaloyl chloride to obtain, after displacement of the remaining chloro group by methanol, the TSAO-T derivative **17** (Fig. 3). This analogue could deliver us information about the importance of the flexibility of methylene groups between the two carbonyls.

### 2.3. Chemical structure analysis

The $^{1}$H and $^{13}$C NMR data supported the integrity of the samples and the structural assignment. Thus, the methylene protons in the *N*-3 substituent for compounds **9**–**16** were observed between 3.0 and 2.0 ppm, whereas the carbonyl carbons resonated at 172–176 ppm. The rest of signals were consistent with the reported data for TSAO-T derivatives.

Because of the existing enolate forms of nucleosidic bases, a competition between *N*-3 and *O*-4 acylation could be observed (even if such a reaction has never been described in literature after *N*-3 functionalism of TSAO-T) [7–10]. The $^{13}$C NMR data demonstrated the regioselectivity of the acylation reaction in favour of *N*-3 position rather than *O*-4. Sekine and coll [21]. carried out a $^{13}$C NMR study of *O*-4 and *N*-3 substituted nucleoside derivatives. In case of a *O*-substitution the chemical shift in $^{13}$C of C-2 is higher (approximately 4 ppm) than that of the starting product, whereas during a *N*-substitution the chemical shift of C-2 carbon is lower (approximately 2 ppm) than that of the starting product. The NMR data for our compounds are in good agreement with these observations as the chemical shifts of C-2 (149.4, 148.3, 148.9 and 149.0 ppm for compounds **3**, **4**, **5** and **6** *versus* 152.0 ppm for the TSAO-T) are lower by approximately 3 ppm. Moreover, the UV spectra analysis was also in favour of *N*-3 acylation rather than *O*-4 acylation. (TSAO-T: $\lambda = 222$ and 264 nm; compound **5**: $\lambda = 224$ and 264 nm).

In the same way, the regioselectivity of the *N*-3 rather than in *N*-4″ acylation was determined by $^{13}$C NMR analysis. The chemical shift of C-4″ of TSAO-T and compounds **3**, **4**, **5** and **6** are similar (150.3 ppm versus 150.2, 150.4, 150.1 and 150.5 ppm) which confirms the acylation taken place in *N*-3 rather than in *N*-4″ position. Indeed in the case of *N*-4″ acylation, the chemical shift of C-4″ is modified. For example after acylation of *N*-4″ with an ureido or a methyloxamoylamino group the chemical shift of C-4″ is 145.58 ppm or 140.48 ppm (between approximately 5 and 10 ppm lower than TSAO-T) [19].

### 2.4. Biological activity

Novel *N*-3 acylated TSAO-T compounds **3**–**6** and **9**–**17** were evaluated for their inhibitory activity against HIV-1($III_B$) and HIV-2(ROD) in human T-lymphocyte CEM cell cultures and against HCV in human Huh-7 hepatoma cell cultures (Table 1).

The stability of compounds **3-6** and **9**–**17** has been studied. After 72 h in PBS at 37 °C no degradation has been observed. As an example of this study, LCMS spectra obtained with compound **10** before, and after incubation are reported (Fig. 4).

#### 2.4.1. Anti-HIV activity

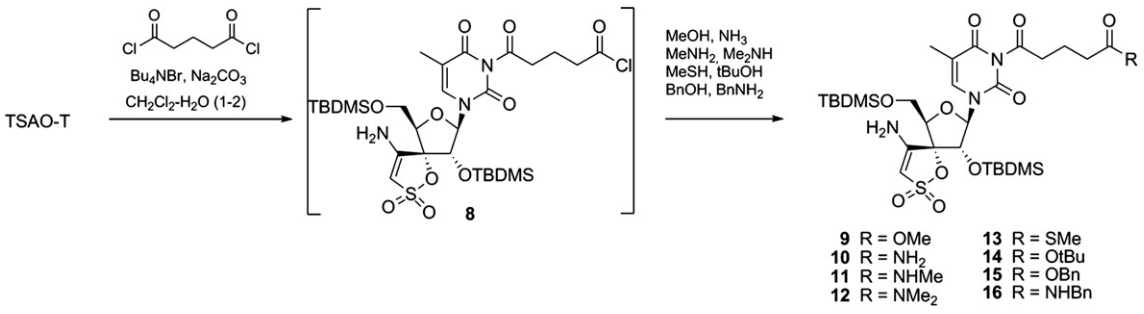
Despite the variety of functional groups of the test compounds, $EC_{50}$ values were all comprised between 0.032 and 0.070 μM. No gain of activity has been observed in comparison with compounds **1** and **2**.

Unfortunately with an $EC_{50}$ of 0.070 μM (compound **4**), the presence of a methyl, less bulky group, does not improve anti-HIV activity compared to TSAO-Boc$^{3}$T (compound **2**, $EC_{50} = 0.023$ μM). So the reduction of the steric constraint does not support the interaction with Thr139 as suggested in a previous work [13]. Nevertheless, compound **4** was less cytostatic ($CC_{50} > 100$ μM).

However if we compare the results obtained for the acylated TSAO-T **3** and **6** with their methylene analogs **18** and **19** synthesized by Camarasa and collaborators [8], anti-HIV activity is 4 times higher for the new compounds (Fig. 5). This outcome is fully consistent with our previous work which has demonstrated that the carbonyl of the Boc group acts as a potential novel pharmacophore for TSAO-T derivatives.

Taking into account the $EC_{50}$ of compound **11** ($EC_{50} = 0.059 \pm 0.003$ μM) and compound **2** ($EC_{50} = 0.023 \pm 0.001$ μM), introduction of a second carbonyl does not improve the antiviral activity (Fig. 6).

None of the compounds in Table 1 showed activity against HIV-2, nor against a mutant HIV-1 strain that contains the TSAO-T-characteristic E138K mutation in its RT (data not shown). These findings confirm HIV-1 RT as the target for the new TSAO-T derivatives.




**Scheme 5.** Glutaryl dichloride route.

**Table 1**
Inhibitory activity of tested compounds against HIV-1 and HIV-2 in CEM cell cultures and HCV.

| Compounds | HIV | | | HCV | |
|---|---|---|---|---|---|
| | $EC_{50}$ (μM)[a] | | $CC_{50}$ (μM)[b] | $EC_{50}$ (μM)[a] | $CC_{50}$ (μM)[b] |
| | HIV-1 | HIV-2 | | | |
| **3** | 0.049 ± 0.003 | ≥4 | 54 ± 8.5 | 9.8 ± 1.0 | >100 |
| **4** | 0.070 ± 0.023 | ≥100 | > 100 | >100 | >100 |
| **5** | 0.052 ± 0.028 | ≥4 | 11 ± 0.71 | 43 ± 2 | >100 |
| **6** | 0.042 ± 0.014 | ≥4 | 11 ± 0.0 | 8.5 ± 0.6 | >100 |
| **9** | 0.054 ± 0.005 | ≥4 | 9.9 ± 0.2 | 11 ± 1 | >100 |
| **10** | 0.038 ± 0.025 | ≥4 | 8.7 ± 0.3 | 9 ± 1 | >100 |
| **11** | 0.059 ± 0.003 | ≥4 | 9.1 ± 0.2 | 12 ± 0.8 | >100 |
| **12** | 0.032 ± 0.016 | ≥4 | 9.5 ± 0.3 | 4.4 ± 0.6 | >100 |
| **13** | 0.037 ± 0.028 | ≥4 | 9.9 ± 0.1 | 7.0 ± 0.9 | >100 |
| **14** | 0.033 ± 0.017 | ≥4 | 11 ± 0.7 | 11 ± 0.9 | >100 |
| **15** | 0.040 ± 0.029 | ≥4 | 9.2 ± 0.8 | 10 ± 0.8 | >100 |
| **16** | 0.052 ± 0.014 | ≥4 | 8.8 ± 0.3 | 7.6 ± 0.7 | >100 |
| **17** | 0.041 ± 0.028 | ≥4 | 14 ± 0.7 | 8.2 ± 0.9 | >100 |
| **TSAO-T**[c] | 0.060 ± 0.030 | >20 | 12 ± 3 | 14 ± 1.8 | >100 |
| **TSAO-m$^3$T**[c] | 0.037 ± 0.001 | >250 | 115 ± 14 | > 100 | >100 |
| **1**[c] | 0.01 ± 0.01 | >10 | 10 → 50 | | |
| **2**[d] | 0.023 ± 0.001 | | 26 ± 1.1 | | |
| **2′-C-Me-C**[e] | | | | 2.4 ± 0.8 | >33 |

[a] 50% Effective concentration.
[b] 50% Cytostatic concentration.
[c] Data taken from ref [8].
[d] Data taken from ref [13].
[e] Data taken from ref [23].

binding pocket and consequential significant rearrangements of RT sub domains upon binding of the inhibitor. It shows that the inhibitor binds inside the NNRTI-binding pocket, assuming a "dragon" shape, and interacts extensively with almost all the pocket residues. A relevant result was that aromatic side chains of Tyr181 and Tyr188 are arranged in a different conformation compared to the commonly observed NNRTI-bound conformation in which both of the aromatic side chains are on one side, forming a wall of the hydrophobic pocket. The hydrophobic valley created by the rearrangement of the two aromatic side chains accommodates the 5′-TBDMS moiety, which establishes hydrophobic interactions with Trp229, Pro95, Tyr183, and Glu138 (p51). The 2′-TBDMS group interacts with Phe227, Tyr318, His235, and Pro236. The ribose and spiro rings are positioned over the β6_β10_β9 sheet. One of the sulfonyl oxygens is hydrogen bonded to with the main chain amino group of Lys103, whereas the other S=O group is involved in a water-mediated interaction network with the enzyme. Finally, the thymine group is positioned between the aromatic side chains of Tyr188 and Phe227, and points toward the flexible loop (residues 214–224) outside the binding pocket.

To shed light into our results, we have performed a docking analysis with the crystal structure of the enzyme in this complex (PDB code 3QO9) as target. As long as a crystallographic water molecule appears near the thymine ring, and hence it could be displaced by a *N*-3 substituent, we carried out the analysis with and without it. While in the first case, the docking was unsuccessful, in the second case the largest and lowest-energy cluster shows that

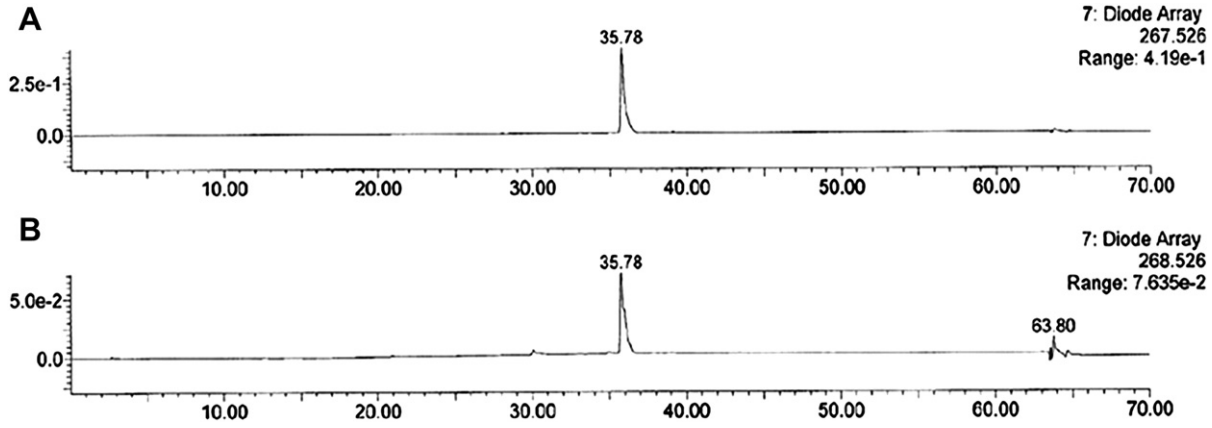



**Fig. 4.** LCMS spectra of compound **10** before incubation (**A**) and after incubation (**B**) in PBS at 37 °C for 72 h.

### 2.4.2. Molecular docking

While the elaboration of this manuscript, the crystal structure of the HIV-1 RT-TSAO-T complex was reported by Das et al. [20]. This structure has revealed an unexpected expansion of the NNRTI molecule **B** is positioned analogously to TSAO-T in the crystal, and the same interactions are observed (Fig. 7). The *N*-3 substituent is accommodated by the space around the 214–224 loop that is outside the NNRTI binding pocket (Fig. 7).

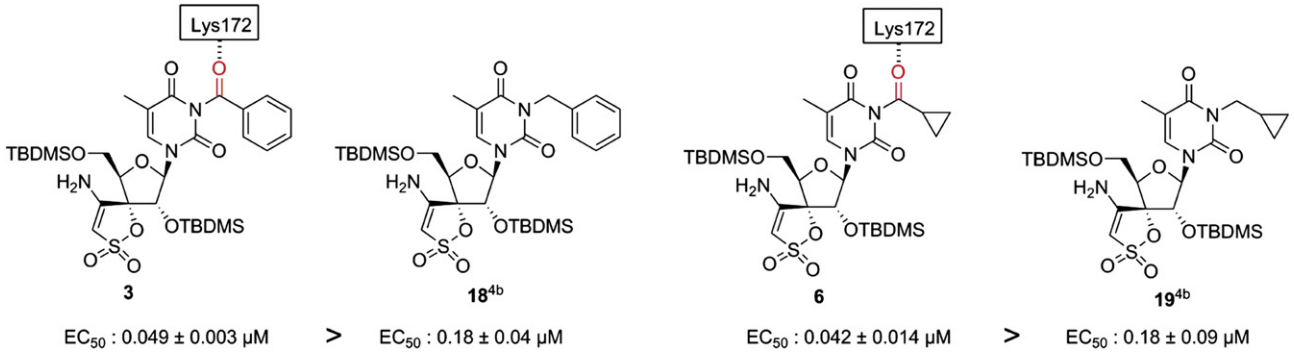



**Fig. 5.** Importance of carbonyl of the Boc group as a potential novel pharmacophore for TSAO-T derivatives (Initial interaction were predicted with Lys 172. The actual interactions with the crystal is with Lys220).

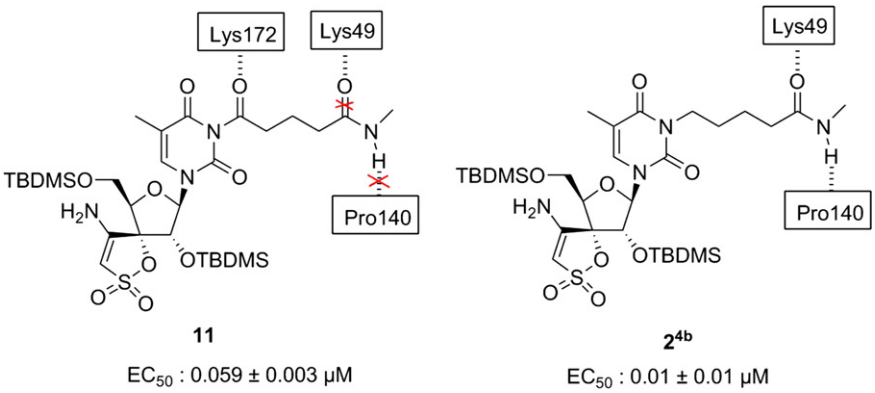



**Fig. 6.** Relative effect of a second carbonyl on the side chain (Initial interaction were predicted with Lys 172. The actual interactions with the crystal is with Lys220).

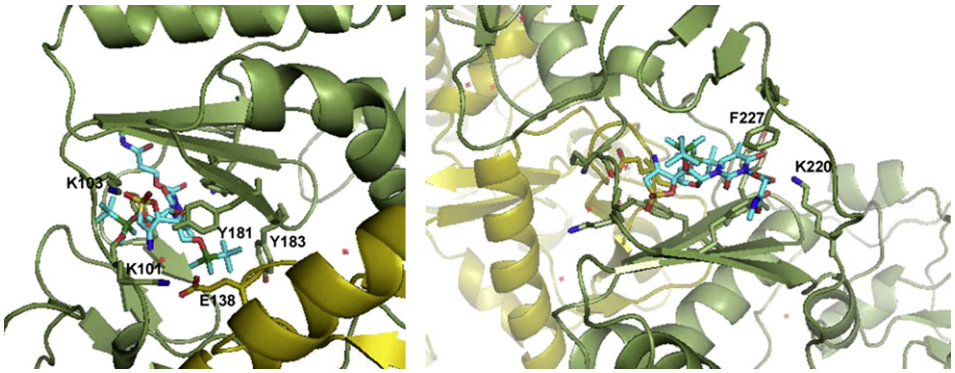



**Fig. 7.** Two views of the predicted binding mode for compound **B** (pdb 3QO9). Residues relevant to the discussion are displayed as sticks with carbon atoms colored according to their subunit. Water molecules are shown as red asterisks. Ligand **B** is displayed as sticks with carbon atoms in cyan. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article. (i.e., if the figure is black and white in the printed version and colour in the web version).

The key hydrophobic interactions with the aromatic side chains (Tyr181, Tyr183 and Tyr188) were not observed in our previous docking study with other crystal structures, due to the expansion of the NNRTI binding pocket and rearrangements of RT subdomains upon binding with this bulky class of inhibitors. Indeed, the new model explains better our current results. The improved activity of **3** and **6** (carbonylic substituents) versus non-carbonylic groups can be accounted by an additional H-bond with Lys220 side chain. Larger *N*-3 substituents (or a second carbonylic group) marginally enhance the potency of the TSAO by gaining interaction outside NNRTI binding pocket, probably only by residual hydrophobic interactions in the large open space around a flexible loop (p66, residues 210–224).

A study of in-depth modeling is running and will be reported in due course.

*2.4.3. HCV results*

Taking into account the $EC_{50}$ values for compounds **3**, **6**, **10**, **13**, **16**, **17** and especially the $EC_{50}$ of compound **12** we can conclude that some TSAO-T derivatives are inhibitory to HCV. Results obtained for compounds **9**, **11**, **14** and **15** were also interesting since their $EC_{50}$'s against HCV were still <15 μM. Compound **5** can be considered as a weak inhibitor with an $EC_{50}$ of 43 μM and compound **4** was the only one to have no activity ($EC_{50}$ > 100 μM). Although it may be unlikely that the derivatives would act through a competitive mechanism against a nucleotide substrate because of their structure (presence of the TBDMS groups in *O*-2′ and *O*-5′) we compared the $EC_{50}$ values of the derivatives with a known HCV inhibitor targeting HCV RNA polymerase, the 2′-*C*-Methyl-Cytidine [24–28], and noticed that the $EC_{50}$ values are very close (4.4 μM for **4** and 2.4 μM for 2′-*C*-Me-C) [23]. Moreover the lack of cytotoxicity of the compounds ($CC_{50}$ > 100 μM) is particularly interesting. However, it is currently unclear whether the anti-HCV activity observed in the virus-infected Huh-7 cell cultures represent a specific antiviral activity of the test compounds or is due to an underlying cytostatic potential of the compounds. Nevertheless, we believe that our findings may represent the starting point of a study to further investigate the potential anti-HCV activity of TSAO-T derivatives.

## 3. Conclusion

In summary, we developed an efficient protocol for the regioselective acylation of TSAO-T at position *N*-3 using acyl chlorides as acylating agents under phase transfer conditions. This methodology has been extended to the preparation of derivatives bearing di-carbonyl motifs designed for cooperative interactions with specific amino acids in the HIV-1 RT. The synthetic strategy involves a two-step, one-pot transformation of TSAO-T by sequential reaction with glutaryl dichloride and a nucleophile and is very well suited for combinatorial approaches.

For HIV, the increased inhibitory activity related to the presence of a *N*-3 acylated not hindered bulky substituent or a *N*-3 di-carbonyl substituent was not observed. The differences in activity demonstrated by Camarasa according to the nature of the substituents were not obtained with our compounds which suggest a more important role of the first carbonyl. These results underlined the fact that only the synthesis of compounds **C** as model of derivatives **B** was performed.

For HCV, biological evaluation revealed that several compounds acted as micromolar inhibitors of HCV JFH1 replication with no visible toxicity in the confluent Huh-7 cell cultures. The compounds prove to have micromolar cytostatic activity in human T-lymphocyte cell cultures. Other biological evaluations are currently sought in our laboratory in view to demonstrate anti-HCV RNA polymerase activity of the novel TSAO derivatives.

## 4. Experimental

### 4.1. Computational methods

#### 4.1.1. Molecular docking

All docking studies were performed with the program AutoDock (version 3.0.5) [29]. As protein target, we selected the crystallographic structure of HIV1-RT in a complex with the non-nucleoside inhibitor α-APA R 95845 (PDB code: 1HNI [30]). The ligand structure were deleted, and polar hydrogens were added. Affinity grid files were generated with AutoGrid (version 3.0). The carboxylic carbon atom of the residue GluB138 was chosen as the center of the grids, and the dimensions of the cubic grid were 90 × 90 × 90 $Å^3$ with grid points separated by 0.300 Å. At each grid point, the receptor's atomic affinity potentials for aliphatic carbon, aromatic carbon, oxygen, nitrogen, sulfur, and hydrogen atoms were computed for intra- and intermolecular energy evaluation of each docked configuration. For simplicity, the Si atoms were assimilated to aliphatic C atoms. Amber united-atom charges and solvation parameters were assigned to the protein with the program ADT (AutoDock Tools, version 1.3a.1).

The ligand coordinates were obtained from ab initio geometry optimizations with Gaussian 98 [31], from structures with $E_4$ envelope puckering for the sugar ring. A common set of atom-centered RHF/6-31G*//RHF/3-21G* charges was then derived by using the RESP methodology [32].

The genetic algorithm implemented in AutoDock was used to generate different RT-bound ligand conformers by randomly changing the torsion angles and overall orientation of the molecule. After docking, the 100 solutions were clustered, and the clusters were ranked by the lowest energy representative of each cluster (see Supporting Information).

#### 4.1.2. Molecular dynamics

The complex obtained from the docking experiments was used as starting structure for the MD simulation. This system was placed into a 25 Å radius spherical cap of 652 TIP3 [33] water molecules centered on C1′ of the ligand. The AMBER (amber99 parm file) all-atom force field [34] was used for the enzyme. For the ligand, bonded and nonbonded parameters were derived, by analogy or through interpolation, from those already present in the AMBER force field, whereas HF/6-31G* RESP charges were used. After minimization, the whole system was partitioned into a mobile and a frozen region. The former consisted of the ligand, all the residues within a radius of 20 Å from the ligand, and the water molecules. After equilibration (300 ps) where positional restraints on both the ligand and the protein were progressively reduced and finally removed, a 2 ns MD simulation (at 298 K) was carried out. A cutoff of 12 Å for nonbonded interactions was used, and SHAKE [35] was applied to all bonds involving hydrogens, and an integration step of 2 fs was used throughout.

The structural stability of the complexes was examined by monitoring the rmsd profiles for the subset of residues that form the walls of the binding site with regard to the crystallographic structure. To examine the energetic stability of the complexes, an MM/PB-SA analysis was performed by using 100 snapshots collected regularly along the last 500 ps of the MD simulation. Accordingly, the stability of each complex was estimated from the addition of the internal energy (including the contributions due to both inhibitor and enzyme and their interaction energy) by means of the AMBER force field, and the solvation free energy by using a Poisson–Boltzmann calculation for the electrostatic component and a surface-area dependent term for the nonelectrostatic contribution. The solute was assigned a dielectric of 1, the solvent dielectric was set to 78.4, and the dielectric boundary was defined using a 1.4 probe sphere and van der Waals radii taken from the AMBER force field. A lattice spacing of 0.67 point/Å was employed. To note that we have not estimate the entropy contribution to binding to save computational time, and because both systems are analogous and directly comparable.

### 4.2. General

Materials and methods. Melting points are uncorrected. Optical rotations were recorded in $CH_2Cl_2$ solution. $^1H$ NMR (300.13 MHz) and $^{13}C$ NMR (75.47 MHz) spectra were recorded in $CDCl_3$, respectively. TLC were performed on Silica F254 and detection by UV light at 254 nm or by charring with cerium molybdate reagent. Mass spectral data were acquired on a WATERS Micromass ZQ spectrometer or a WATERS Micromass Q-TOFF spectrometer. HPLC analysis was performed on Waters-Breeze (2487 Dual l Absorbance Detector and 1525 Binary HPLC Pump) with Prevail C18 column. Column chromatography was effected on Silica Gel 60 (230 mesh). Cyclohexane and ethyl acetate were distilled before use. TSAO-T was synthesized according reference [36].

### 4.3. General methods

#### 4.3.1. General procedure for the synthesis of 3-N-monocarbonyl TSAO-T derivatives (**3**–**6**)

TSAO-T (1 mmol), $Na_2CO_3$ (8 mmol) and $Bu_4NBr$ (0.1 mmol) were dissolved in a mixture of $CH_2Cl_2$ and water (1/2 V/V). Acylated reactant (1.2 mmol) was added to the mixture with vigorous stirring at room temperature until disappearance of the starting material (TLC monitoring). Water and $CH_2Cl_2$ were added; the organic phase was separated, washed with water (3 times), dried ($Na_2SO_4$) and concentrated. The crude product was purified by flash chromatography followed by crystallization from water to give the final product.

*4.3.1.1. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-benzoylthymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**3**).* According to the general procedure; TSAO-T (50 mg, 8.48 × $10^{-2}$ mmol), $Na_2CO_3$ (72 mg, 0.679 mmol) and $Bu_4NBr$ (2.7 mg, 8.48 × $10^{-3}$ mmol) in $CH_2Cl_2$ (1 mL) and water (2 mL) were treated with benzoyl chloride (11.8 μL, 0.101 mmol). The crude product was purified by flash chromatography (EtOAc/cyclohexane, 3/7) followed by crystallization from water to give **3** (45 mg, 76%) as a white solid. $R_f$ = 0.62 (EtOAc/Cyclohexane : 5/5); Mp = 171–172 °C; $[\alpha]_D^{21} = -38$ (*c* 0.1, $CH_2Cl_2$); $^1H$ NMR ($CDCl_3$; 300 MHz) : δ 7.94 (m, 2H, $COC_6H_5$), 7.69 (m, 1H, $COC_6H_5$), 7.52 (m, 2H, $COC_6H_5$), 7.36 (s, 1H, H-6), 5.96 (d, 1H, $J_{1',2'}$ = 7.9 Hz, H-1′), 5.63 (s, 2H, $NH_2$), 5.60 (s, 1H, H-3′), 4.62 (d, 1H, H-2′), 4.31 (m, 1H, H-4′), 4.02 (dd, $J_{5'a,4'}$ = 2.6 Hz, $J_{5'a,5'b}$ = 12.4 Hz, 1H, H-5′a), 3.90 (dd, $J_{5'b,4'}$ = 2.6 Hz, 1H, H-5′b), 2.01 (s, 3H, $CH_3$ base), 0.99 (s, 9H, $SiC(CH_3)_3$), 0.88 (s, 9H, $SiC(CH_3)_3$), 0.22 (s, 3H, $Si(CH_3)_2$), 0.11 (s, 3H, $Si(CH_3)_2$), 0.10 (s, 3H, $Si(CH_3)_2$), 0.04 (s, 3H, $Si(CH_3)_2$); $^{13}C$ NMR ($CDCl_3$; 75 MHz) : δ 168.2 (CO), 162.5 (C-4), 150.5 (C-4″), 149.4 (C-2), 135.2 (C-6), 131.4 ($C^{IV}C_6H_5$), 135.9, 130.5, 129.1 ($C_6H_5$), 111.9 (C-5), 92.9 (C-3″), 91.9 (C-3′), 87.9 (C-1′), 83.7 (C-4′), 74.4 (C-2′), 62.0 (C-5′), 26.0, 25.4 (2x $SiC(CH_3)_3$), 18.4, 17.9 (2x $SiC(CH_3)_3$), 12.3 ($CH_3$

base), −4.4, −4.8, −5.0, −5.6 (2x $Si(CH_3)_2$). HRMS: $C_{31}H_{47}N_3O_9S$-$Si_2Na$ calcd. 716.2469, found 716.2484.

*4.3.1.2. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(methoxycarbonyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**4**).* According to the general procedure TSAO-T (50 mg, 8.48 × $10^{-2}$ mmol), $Na_2CO_3$ (72 mg, 0.679 mmol) and $Bu_4NBr$ (2.7 mg, 8.48 × $10^{-3}$ mmol) in $CH_2Cl_2$ (1 mL) and water (2 mL) were treated with methyl chloroformate (10.4 μL, 0.110 mmol). The crude product was purified by HPLC ($CH_3CN/H_2O$ 0.1%TFA) followed by crystallization from water to give **4** (23 mg, 42%) as a white solid. $R_f$ = 0.69 ($CH_2Cl_2$/MeOH : 98/2); Mp = 229–230 °C; $[\alpha]_D^{21}$ = −20 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.26 (s, 1H, H-6), 5.94 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.66 (s, 1H, H-3″), 5.55 (s, 2H, $NH_2$), 4.55 (d, 1H, H-2′), 4.38 (m, 1H, H-4′), 4.07 (s, 3H, $OCH_3$), 4.04 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.4 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 2.3 Hz, 1H, H-5′b), 2.01 (s, 3H, $CH_3$ base), 0.99 (s, 9H, $SiC(CH_3)_3$), 0.87 (s, 9H, $SiC(CH_3)_3$), 0.24 (s, 3H, $Si(CH_3)_2$), 0.22 (s, 3H, $Si(CH_3)_2$), 0.08 (s, 3H, $Si(CH_3)_2$), −0.03 (s, 3H, $Si(CH_3)_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 160.8 (C-4), 150.2 (CO), 150.1 (C-4′), 148.4 (C-2), 135.2 (C-6), 111.5 (C-5), 93.5 (C-3′), 92.1 (C-3′), 87.6 (C-1′), 83.8 (C-4′), 74.7 (C-2′), 62.1 (C-5′), 56.2 ($OCH_3$), 26.0, 25.2 (2x $SiC(CH_3)_3$), 18.4, 17.8 (2x $SiC(CH_3)_3$), 12.3 ($CH_3$ base), −4.5, −4.9, −5.3, −5.7 (2x $Si(CH_3)_2$). HRMS: $C_{26}H_{45}N_3O_{10}S$-$Si_2Na$ calcd. 670.2262, found 670.2237.

*4.3.1.3. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(bromobutanoyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**5**).* According to the general procedure TSAO-T (104 mg, 0.176 mmol), $Na_2CO_3$ (150 mg, 1.41 mmol) and $Bu_4NBr$ (5.7 mg, 1.76 × $10^{-2}$ mmol) in $CH_2Cl_2$ (7 mL) and water (14 mL) were treated with 4-bromobutanoyl chloride (23 μL, 0.196 mmol). The crude product was purified by HPLC ($MeOH/H_2O$) followed by crystallization from water to give 5 (91 mg, 70%) as a white solid. $R_f$ = 0.67 ($CH_2Cl_2$/MeOH : 98/2); Mp = 192–193 °C; $[\alpha]_D^{21}$ = −14 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.23 (s, 1H, H-6), 5.94 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1′), 5.63 (s, 2H, $NH_2$), 5.56 (s, 1H, H-3″), 4.48 (d, 1H, H-2′), 4.34 (m, 1H, H-4′), 3.98 (dd, $J_{5'a,4'}$ = 2.6 Hz, $J_{5'a,5'b}$ = 12.4 Hz, 1H, H-5′a), 3.87 (dd, $J_{5'b,4'}$ = 2.0 Hz, 1H, H-5′b), 3.49 (t, $J_{a,b}$ = 6.4 Hz, 2H, $CH_{2a}$), 2.98 (t, $J_{c,b}$ = 6.8 Hz, 2H, $CH_{2c}$), 2.30 (m, 2H, $CH_{2b}$), 1.96 (s, 3H, $CH_3$ base), 0.95 (s, 9H, $SiC(CH_3)_3$), 0.83 (s, 9H, $SiC(CH_3)_3$), 0.20 (s, 3H, $Si(CH_3)_2$), 0.19 (s, 3H, $Si(CH_3)_2$), 0.05 (s, 3H, $Si(CH_3)_2$), −0.07 (s, 3H, $Si(CH_3)_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 174.7 (CO), 161.8 (C-4), 150.2 (C-4″), 148.9 (C-2), 135.0 (C-6), 112.0 (C-5), 93.4 (C-3″), 92.4 (C-3′), 86.7(C-1′), 83.7 (C-4′), 74.8 (C-2′), 62.1 (C-5′), 38.9, 31.8, 26.7 (3x $CH_2$), 26.0, 25.3 (2x $SiC(CH_3)_3$), 18.4, 17.8 (2x $SiC(CH_3)_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.2, −5.6 (2x $Si(CH_3)_2$). HRMS: $C_{28}H_{48}BrN_3O_9SSi_2Na$ calcd. 760.1731, found 760.1719.

*4.3.1.4. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(cyclopropanoyl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**6**).* According procedure described in. 4.3.3 and using a 0.23 M concentration of sodium carbonate, a mixture of the desired 4-bromobutanoyl derivative **5** and of the cyclopropylcarbonyl product **6** was obtained in a ratio 76/24 determined by NMR analysis on the crude mixture. A pure sample of **6** was obtained after HPLC ($MeOH/H_2O$) purification. $R_f$ = 0.53 ($CH_2Cl_2$/MeOH : 98/2); Mp = 199–201 °C; $[\alpha]_D^{21}$ = −40 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.27 (s, 1H, H-6), 5.98 (d, 1H, $J_{1',2'}$ = 8.0 Hz, H-1′), 5.67 (s, 2H, $NH_2$), 5.63 (s, 1H, H-3″), 4.56 (d, 1H, H-2′), 4.37 (m, 1H, H-4′), 4.03 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.4 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 2.2 Hz, 1H, H-5′b), 2.03 (s, 3H, $CH_3$ base), 1.99 (m, 1H, CH), 1.42 (m, 1H, $CH_2$), 1.22 (m, 1H, $CH_2$), 1.00 (s, 9H, $SiC(CH_3)_3$), 0.85 (s, 9H, $SiC(CH_3)_3$), 0.24 (s, 3H, $Si(CH_3)_2$), 0.23 (s, 3H, $Si(CH_3)_2$), 0.09 (s, 3H, $Si(CH_3)_2$), −0.02 (s, 3H, $Si(CH_3)_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 176.4 (CO), 161.7 (C-4), 150.4 (C-4″), 149.0 (C-2), 135.4 (C-6), 111.9 (C-5), 93.3 (C-3″), 92.3 (C-3′), 87.2 (C-1′), 83.7 (C-4′), 74.7 (C-2′), 62.1 (C-5′), 26.0, 25.3 (2x $SiC(CH_3)_3$), 20.1 (CH), 18.4, 17.9 (2x $SiC(CH_3)_3$), 13.0, 13.1 (2x $CH_2$), 12.3 ($CH_3$ base), −4.5, −4.8, −5.2, −5.7 (2x $Si(CH_3)_2$). HRMS: $C_{28}H_{47}N_3O_9S$-$Si_2Na$ calcd. 680.2469, found 680.2485.

*4.3.2. General procedure for the synthesis of 3-N-dicarbonyl TSAO-T derivatives (**9**–**16**)*

TSAO-T (1 mmol), $Na_2CO_3$ (8 mmol) and $Bu_4NBr$ (0.1 mmol) were dissolved in a mixture of $CH_2Cl_2$ and water (1/2 V/V). Glutaryl dichloride (3.5 mmol) was added to the mixture with vigorous stirring at 8 °C until disappearance of the starting material (mass spectrometry monitoring). After 15 min the reaction mixture was quenched with appropriate nucleophile. Vigorous stirring was continued at room temperature between 5 and 15 min. Water and $CH_2Cl_2$ were added; the organic phase was separated, washed with water (3 times), dried ($Na_2SO_4$) and concentrated. The crude product was purified by HPLC followed by crystallization from water to give the final product.

*4.3.2.1. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(O-methoxyglutaryl)thymine]-3′spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**9**).* According to the general procedure TSAO-T (64.7 mg, 0.109 mmol), $Na_2CO_3$ (93 mg, 0.878 mmol) and $Bu_4NBr$ (3.5 mg, 1.09 × $10^{-2}$ mmol) in $CH_2Cl_2$ (2 mL) and water (4 mL) were treated with glutaryl dichloride (42 μL, 0.329 mmol) and quenched with MeOH (4 mL). The crude product was purified by HPLC ($MeOH/H_2O$) followed by crystallization from water to give **9** (51 mg, 36%) as a white solid. $R_f$ = 0.69 (EtOAc/Cyclohexane : 5/5); Mp = 165–167 °C; $[\alpha]_D^{21}$ = −19 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.26 (s, 1H, H-6), 5.97 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1′), 5.67 (s, 1H, H-3″), 5.60 (s, 2H, $NH_2$), 4.52 (d, 1H, H-2′), 4.37 (m, 1H, H-4′), 4.03 (dd, $J_{5'a,4'}$ = 2.6 Hz, $J_{5'a,5'b}$ = 12.4 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 2.1 Hz, 1H, H-5′b), 3.71 (s, 3H, $OCH_3$), 2.91 (t, $J_{a,b}$ = 6.9 Hz, 2H, $CH_{2a}$), 2.49 (t, $J_{c,b}$ = 7.4 Hz, 2H, $CH_{2c}$), 2.10 (m, 2H, $CH_{2b}$), 2.00 (s, 3H, $CH_3$ base), 1.00 (s, 9H, $SiC(CH_3)_3$), 0.87 (s, 9H, $SiC(CH_3)_3$), 0.24 (s, 3H, $Si(CH_3)_2$), 0.23 (s, 3H, $Si(CH_3)_2$), 0.09 (s, 3H, $Si(CH_3)_2$), −0.03 (s, 3H, $Si(CH_3)_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 174.9 (CO), 173.2 (CO), 161.8 (C-4), 150.2 (C-4″), 148.9 (C-2), 135.0 (C-6), 111.9 (C-5), 93.4 (C-3″), 92.2 (C-3′), 86.9 (C-1′), 83.7 (C-4′), 74.7 (C-2′), 62.1 (C-5′), 51.6 ($OCH_3$), 39.4, 32.3 (2x $CH_2$), 26.0, 25.2 (2x $SiC(CH_3)_3$), 18.8 ($CH_2$), 18.4, 17.8 (2x $SiC(CH_3)_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.2, −5.6 (2x $Si(CH_3)_2$). HRMS: $C_{30}H_{51}N_3O_{11}SSi_2Na$ calcd.740.2681, found 740.2671.

*4.3.2.2. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(aminoglutaryl)thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**10**).* According to the general procedure TSAO-T (61.8 mg, 0.104 mmol), $Na_2CO_3$ (89 mg, 0.839 mmol) and $Bu_4NBr$ (3.4 mg, 1.04 $10^{-2}$ mmol) in $CH_2Cl_2$ (5 mL) and water (10 mL) were treated with glutaryl dichloride (47 μL, 0.367 mmol) and quenched with $NH_3$ (7 N MeOH) (90 μL, 0.629 mmol). The crude product was purified by HPLC ($MeOH/H_2O$) followed by crystallization from water to give **10** (51 mg, 70%) as a white solid. $R_f$ = 0.10 (EtOAc/Cyclohexane : 5/5); Mp = 114–115 °C; $[\alpha]_D^{21}$ = −17 (c 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.28 (s, 1H, H-6), 6.01 (d, 1H, $J_{1',2'}$ = 8.1 Hz, H-1′), 5.83 (s, 1H, $CONH_2$), 5.68 (s, 1H, H-3″), 5.61 (s, 2H, $NH_2$), 5.45 (s, 1H, $CONH_2$), 4.48 (d, 1H, H-2′), 4.39 (m, 1H, H-4′), 4.03 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.5 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 1.9 Hz, 1H, H-5′b), 2.93 (t, $J_{a,b}$ = 6.2 Hz, 2H, $CH_{2a}$), 2.41 (t, $J_{c,b}$ = 7.1 Hz, 2H, $CH_{2c}$), 2.10 (m, 2H, $CH_{2b}$), 2.00 (s, 3H, $CH_3$ base), 1.00 (s, 9H, $SiC(CH_3)_3$), 0.83 (s, 9H, $SiC(CH_3)_3$), 0.25 (s, 3H, $Si(CH_3)_2$), 0.24 (s, 3H, $Si(CH_3)_2$), 0.09 (s, 3H, $Si(CH_3)_2$), −0.03 (s, 3H, $Si(CH_3)_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 175.3 (CO), 174.8 (CO), 161.9 (C-4),

150.2 (C-4″), 149.1 (C-2), 134.8 (C-6), 112.0 (C-5), 93.4 (C-3″), 92.5 (C-3′), 86.2 (C-1′), 83.8 (C-4′), 74.9 (C-2′), 62.2 (C-5′), 39.3, 33.8 (2x $CH_2$), 26.0, 25.2 (2x SiC*(CH$_3$)$_3$*), 19.4 ($CH_2$), 18.4, 17.8 (2x SiC(CH$_3$)$_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.2, −5.7 (2x Si(CH$_3$)$_2$). HRMS: $C_{29}H_{50}N_4O_{10}SSi_2Na$ calcd. 725.2684, found 725.2679.

*4.3.2.3. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(N-methylaminoglutaryl)thymine]- 3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**11**).* According to the general procedure TSAO-T (64.9 mg, 0.110 mmol), $Na_2CO_3$ (93 mg, 0.881 mmol) and $Bu_4NBr$ (3.5 mg, 1.1 $10^{-2}$ mmol) in $CH_2Cl_2$ (2 mL) and water (4 mL) were treated with glutaryl dichloride (56.2 μL, 0.440 mmol) and quenched with $MeNH_2$ (33% EtOH) (108 μL, 0.851 mmol). The crude product was purified by flash chromatography (EtOAc/cyclohexane, 7/3) followed by crystallization from water to give **11** (55 mg, 70%) as a white solid. $R_f$ = 0.21 (EtOAc/Cyclohexane :5/5); Mp = 118–120 °C; $[\alpha]_D^{21}$ = −22 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.30 (s, 1H, H-6), 6.04 (d, 1H, $J_{1',2'}$ = 8.2 Hz, H-1′), 5.96 (s, 1H, NH), 5.82 (s, 2H, $NH_2$), 5.69 (s, 1H, H-3″), 4.44 (d, 1H, H-2′), 4.38 (m, 1H, H-4′), 4.02 (dd, $J_{5'a,4'}$ = 2.6 Hz, $J_{5'a,5'b}$ = 12.6 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 1.6 Hz, 1H, H-5′b), 2.88 (t, $J_{a,b}$ = 6.3 Hz, 2H, $CH_{2a}$), 2.81 (d, $J_{NH,CH3}$ = 4.8 Hz, 3H, $CH_3NH$), 2.34 (t, $J_{c,b}$ = 7.2 Hz, 2H, $CH_{2c}$), 2.10 (m, 2H, $CH_{2b}$), 1.99 (s, 3H, $CH_3$ base), 1.00 (s, 9H, SiC(CH$_3$)$_3$), 0.85 (s, 9H, SiC(CH$_3$)$_3$), 0.24 (s, 3H, Si(CH$_3$)$_2$), 0.23 (s, 3H, Si(CH$_3$)$_2$), 0.09 (s, 3H, Si(CH$_3$)$_2$), −0.04 (s, 3H, Si(CH$_3$)$_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 175.4 (CO), 172.9 (CO), 161.9 (C-4), 150.3 (C-4″), 149.1 (C-2), 134.6 (C-6), 112.0 (C-5), 93.1 (C-3″), 92.8 (C-3′), 86.7 (C-1′), 83.8 (C-4′), 75.0 (C-2′), 62.2 (C-5′), 39.3, 34.7 (2x $CH_2$), 26.3 ($CH_3NH$), 26.0, 25.2 (2x SiC*(CH$_3$)$_3$*), 19.7 ($CH_2$), 18.4, 17.8 (2x SiC(CH$_3$)$_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.2, −5.6 (2x Si(CH$_3$)$_2$). HRMS: $C_{30}H_{52}N_4O_{10}SSi_2Na$ calcd. 739.2840, found 739.2837.

*4.3.2.4. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(N,N- dimethylaminoglutaryl)thymine]-3′-spiro-5″-(4″-amino-1″, 2″-oxathiole-2″,2″-dioxide) (**12**).* According to the general procedure TSAO-T (55.2 mg, 9.6 $10^{-2}$ mmol), $Na_2CO_3$ (79 mg, 0.749 mmol) and $Bu_4NBr$ (3.0 mg, 9.6 $10^{-3}$ mmol) in $CH_2Cl_2$ (5 mL) and water (10 mL) were treated with glutaryl dichloride (41.8 μL, 0.327 mmol) and quenched with dimethylamine (40% in water) (70.5 μL, 0.562 mmol). The crude product was purified by HPLC ($MeOH/H_2O$) followed by crystallization from water to give **12** (45 mg, 66%) as a white solid. $R_f$ = 0.10 (EtOAc/Cyclohexane : 5/5); Mp = 168–170 °C; $[\alpha]_D^{21}$ = −23 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.28 (s, 1H, H-6), 6.02 (d, 1H, $J_{1',2'}$ = 8.2 Hz, H-1′), 5.76 (s, 2H, $NH_2$), 5.67 (s, 1H, H-3″), 4.47 (d, 1H, H-2′), 4.37 (m, 1H, H-4′), 4.02 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.5 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 1.7 Hz, 1H, H-5′b), 3.04 (s, 3H, $NCH_3$), 2.96 (s, 3H, $NCH_3$), 2.92 (m, 2H, $CH_{2a}$), 2.49 (t, $J_{c,b}$ = 7.1 Hz, 2H, $CH_{2c}$), 2.09 (m, 2H, $CH_{2b}$), 1.99 (s, 3H, $CH_3$ base), 0.99 (s, 9H, SiC(CH$_3$)$_3$), 0.86 (s, 9H, SiC(CH$_3$)$_3$), 0.24 (s, 3H, Si(CH$_3$)$_2$), 0.23 (s, 3H, Si(CH$_3$)$_2$), 0.07 (s, 3H, Si(CH$_3$)$_2$), −0.04 (s, 3H, Si(CH$_3$)$_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 175.6 (CO), 172.0 (CO), 161.8 (C-4), 150.3 (C-4″), 149.0 (C-2), 134.7 (C-6), 111.9 (C-5), 93.2 (C-3″), 92.5 (C-3′), 86.2 (C-1′), 83.7 (C-4′), 74.9 (C-2′), 62.1 (C-5′), 39.6 ($CH_2$), 37.2 ($CH_3N$), 35.4 ($CH_3N$), 31.4 ($CH_2$), 26.0, 25.2 (2x SiC*(CH$_3$)$_3$*), 19.1 ($CH_2$), 18.4, 17.8 (2x SiC(CH$_3$)$_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.2, −5.6 (2x Si(CH$_3$)$_2$). HRMS: $C_{31}H_{54}N_4O_{10}SSi_2Na$ calcd. 753.2997, found 753.3003.

*4.3.2.5. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(thiomethylglutaryl) thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**13**).* According to the general procedure TSAO-T (84 mg, 0.14 mmol), $Na_2CO_3$ (121 mg, 1.14 mmol) and $Bu_4NBr$ (4.6 mg, 1.42 × $10^{-2}$ mmol) in $CH_2Cl_2$ (7 mL) and water (14 mL) were treated with glutaryl dichloride (64 μL, 0.49 mmol) and quenched with MeSNa (60 mg, 0.85 mmol). The crude product was purified by HPLC to give **13** (80 mg, 76%) as a white solid. $R_f$ = 0.73 ($CH_2Cl_2$/MeOH : 9.8/0.2); Mp = 171–172 °C; $[\alpha]_D^{21}$ : −24 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.25 (s, 1H, H-6), 5.95 (d, $J_{1',2'}$ = 8.0 Hz, 1H, H-1′), 5.66 (s, 1H, H-3″), 5.57 (s, 2H, $NH_2$), 4.53 (d, 1H, H-2′), 4.37 (m, 1H, H-4′), 4.03 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.5 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 2.2 Hz, 1H, H-5′b), 2.90 (t, $J_{a,b}$ = 6.9 Hz, 2H, $CH_{2a}$), 2.73 (t, $J_{c,b}$ = 7.3 Hz, 2H, $CH_{2c}$), 2.33 (s, 3H, $SCH_3$), 2.13 (m, 2H, $CH_{2b}$), 2.00 (s, 3H, $CH_3$ base), 1.00 (s, 9H, SiC(CH$_3$)$_3$), 0.88 (s, 9H, SiC(CH$_3$)$_3$), 0.24 (s, 3H, Si(CH$_3$)$_2$), 0.23 (s, 3H, Si(CH$_3$)$_2$), 0.09 (s, 3H, Si(CH$_3$)$_2$), −0.02 (s, 3H, Si(CH$_3$)$_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 198.9 (*C*OSCH$_3$), 174.8 (*C*OCH$_2$), 161.8 (C-4), 150.3 (C-4″), 149.0 (C-2), 135.2 (C-6), 112.0 (C-5), 93.5 (C-3″), 92.2 (C-3′), 87.1 (C-1′), 83.8 (C-4′), 74.7 (C-2′), 62.1 (C-5′), 42.0, 39.4 ($CH_2$), 26.0, 25.3 (SiC*(CH$_3$)$_3$*), 19.4 ($CH_2$), 18.4, 17.9 (SiC(CH$_3$)$_3$), 12.2 ($CH_3$ base), 11.6 ($SCH_3$), −4.5, −4.8, −5.2, −5.7 (Si(CH$_3$)$_2$); HRMS : $C_{30}H_{51}N_3O_{10}S_2Si_2Na$ calcd. 756.2452, found 756.2444.

*4.3.2.6. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(tert-butoxyglutaryl) thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**14**).* According to the general procedure TSAO-T (103 mg, 0.17 mmol), $Na_2CO_3$ (148 mg, 1.39 mmol) and $Bu_4NBr$ (5.6 mg, 1.7 × $10^{-2}$ mmol) in $CH_2Cl_2$ (10 mL) and water (20 mL) were treated with glutaryl dichloride (78 μL, 0.61 mmol) and quenched with *t*-BuOH (1 mL, 0.01 mol). The crude product was purified by HPLC to give **14** (82 mg, 62%) as a white solid. $R_f$ = 0.80 (EtOAc/Cyclohexane : 5/5); Mp = 179–180 °C; $[\alpha]_D^{21}$ : −19 (*c* 0.1, $CH_2Cl_2$); $^1$H NMR ($CDCl_3$; 300 MHz) : δ 7.25 (s, 1H, H-6), 5.95 (d, $J_{1',2'}$ = 8.0 Hz, 1H, H-1′), 5.66 (s, 1H, H-3″), 5.59 (s, 2H, $NH_2$), 4.53 (d, 1H, H-2′), 4.36 (m, 1H, H-4′), 4.03 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.5 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 2.3 Hz, 1H, H-5′b), 2.89 (t, $J_{a,b}$ = 6.9 Hz, 2H, $CH_{2a}$), 2.38 (t, $J_{c,b}$ = 7.4 Hz, 2H, $CH_{2c}$), 2.05 (m, 2H, $CH_{2b}$), 2.00 (s, 3H, $CH_3$ base), 1.47 (s, 9H, OtBu), 0.99 (s, 9H, SiC(CH$_3$)$_3$), 0.87 (s, 9H, SiC(CH$_3$)$_3$), 0.25 (s, 3H, Si(CH$_3$)$_2$), 0.23 (s, 3H, Si(CH$_3$)$_2$), 0.09 (s, 3H, Si(CH$_3$)$_2$), −0.03 (s, 3H, Si(CH$_3$)$_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 175.0 (CO), 172.1 (CO), 161.8 (C-4), 150.3 (C-4″), 148.9 (C-2), 135.1 (C-6), 111.9 (C-5), 93.4 (C-3″), 92.1 (C-3′), 87.1 (C-1′), 83.7 (C-4′), 74.7 (C-2′), 62.1 (C-5′), 39.5, 33.9 ($CH_2$), 28.1 (O*t*-Bu), 26.0, 25.2 (SiC*(CH$_3$)$_3$*), 18.9 ($CH_2$), 18.4, 17.8 (SiC(CH$_3$)$_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.2, −5.7 (Si(CH$_3$)$_2$); HRMS : $C_{33}H_{57}N_3O_{11}SSi_2Na$ calcd. 782.3150, found 782.3161.

*4.3.2.7. [1-[2′,5′-Bis-O-tert-butyldimethylsilyl-β-d-ribofuranosyl]-3-N-(benzyloxyglutaryl) thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**15**).* According to the general procedure TSAO-T (51 mg, 8.6 × $10^{-2}$ mmol), $Na_2CO_3$ (73 mg, 0.68 mmol) and $Bu_4NBr$ (2.8 mg, 8.6 × $10^{-3}$ mmol) in $CH_2Cl_2$ (5 mL) and water (10 mL) were treated with glutaryl dichloride (38 μL, 0.30 mmol) and quenched with BnOH (53 μL, 0.51 mmol). The crude product was purified by HPLC to give **15** (35 mg, 51%). $R_f$ = 0.29 (EtOAc/Cyclohexane : 5/5); Mp = 185–187 °C; $[\alpha]_D^{21}$ = −33 (*c* 0.1, $CH_2Cl_2$); NMR $^1$H ($CDCl_3$; 300 MHz) : δ 7.39–7.38 (m, 5H, $C_5H_6$), 7.25 (s, 1H, H-6), 5.95 (d, $J_{1',2'}$ = 8.0 Hz, 1H, H-1′), 5.65 (s, 1H, H-3″), 5.57 (s, 2H, $NH_2$), 5.16 (s, 2H, *CH$_2$*C$_6$H$_5$), 4.53 (d, 1H, H-2′), 4.36 (m, 1H, H-4′), 4.03 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.5 Hz, 1H, H-5′a), 3.92 (dd, $J_{5'b,4'}$ = 2.2 Hz, 1H, H-5′b), 2.92 (t, $J_{a,b}$ = 6.9 Hz, 2H, $CH_{2a}$), 2.55 (t, $J_{c,b}$ = 7.4 Hz, 2H, $CH_{2c}$), 2.12 (m, 2H, $CH_{2b}$), 2.00 (s, 3H, $CH_3$ base), 0.99 (s, 9H, SiC(CH$_3$)$_3$), 0.87 (s, 9H, SiC(CH$_3$)$_3$), 0.24 (s, 3H, Si(CH$_3$)$_2$), 0.22 (s, 3H, Si(CH$_3$)$_2$), 0.09 (s, 3H, Si(CH$_3$)$_2$), −0.03 (s, 3H, Si(CH$_3$)$_2$); $^{13}$C NMR ($CDCl_3$; 75 MHz) : δ 174.9 (CO), 172.6 (CO), 161.8 (C-4), 150.2 (C-4″), 148.9 (C-2), 135.1 (C-6), 128.6, 128.2, 128.1 ($C_6H_5$), 111.9 (C-5), 93.4 (C-3″), 92.1 (C-3′), 87.1 (C-1′), 83.7 (C-4′), 74.7 (C-2′), 66.3 (*CH$_2$*C$_5$H$_6$), 62.1 (C-5′), 39.4, 32.5 ($CH_2$), 26.0, 25.2 (SiC*(CH$_3$)$_3$*), 18.8 ($CH_2$), 18.4, 17.8 (SiC(CH$_3$)$_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.2, −5.6 (Si(CH$_3$)$_2$); HRMS : $C_{36}H_{55}N_3O_{11}SSi_2Na$ calcd. 816.2994, found 816.2969.

*4.3.2.8. [1-[2′,5′-Bis-O-tert-butyldiméthylsilyl-β-d-ribofuranosyl]-3-N-(N-benzylaminoglutaryl) thymine]-3′-spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**16**).* According to the general procedure TSAO-T (53 mg, $9.0 \times 10^{-2}$ mmol), $Na_2CO_3$ (76 mg, 0.72 mmol) and $Bu_4NBr$ (2.9 mg, $9.0 \times 10^{-3}$ mmol) in $CH_2Cl_2$ (5 mL) and water (10 mL) were treated with glutaryl dichloride (40 μL, 0.31 mmol) and quenched with $BnNH_2$ (59 μL, 0.54 mmol). The crude product was purified by HPLC to give **16** (40 mg, 56%). $R_f = 0.23$ (EtOAc/Cyclohexane : 5/5); Mp = 120–122 °C; $[\alpha]_D^{21}$ : −21 (*c* 0.1, $CH_2Cl_2$); $^1H$ NMR ($CDCl_3$; 300 MHz) : δ 7.36–7.29 (m, 6H, $C_5H_6$ et H-6), 6.20 (t, *J* = 5.6 Hz, 1H, *NHC*$_5H_6$,), 6.01 (d, $J_{1',2'}$ = 8.1 Hz, 1H, H-1′), 5.71 (s, 2H, $NH_2$), 5.67 (s, 1H, H-3″), 4.47–4.43 (m, 3H, *CH*$_2C_6H_5$ et H-2′), 4.37 (m, 1H, H-4′), 4.02 (dd, $J_{5'a,4'}$ = 2.7 Hz, $J_{5'a,5'b}$ = 12.5 Hz, 1H, H-5′a), 3.91 (dd, $J_{5'b,4'}$ = 1.8 Hz, 1H, H-5′b), 2.89 (t, $J_{a,b}$ = 6.2 Hz, 2H, $CH_{2a}$), 2.39 (t, $J_{c,b}$ = 7.2 Hz, 2H, $CH_{2c}$), 2.09 (m, 2H, $CH_{2b}$), 1.96 (s, 3H, $CH_3$ base), 1.00 (s, 9H, SiC$(CH_3)_3$), 0.86 (s, 9H, SiC$(CH_3)_3$), 0.24 (s, 3H, Si$(CH_3)_2$), 0.23 (s, 3H, Si$(CH_3)_2$), 0.08 (s, 3H, Si$(CH_3)_2$), −0.04 (s, 3H, Si$(CH_3)_2$); $^{13}C$ NMR ($CDCl_3$; 75 MHz) : δ 175.3 (CO), 172.0 (CO), 161.8 (C-4), 150.2 (C-4″), 149.0 (C-2), 134.7 (C-6), 128.7, 127.8127.5 ($C_6H_5$), 112.0 (C-5), 93.4 (C-3″), 92.6 (C-3′), 86.0 (C-1′), 83.8 (C-4′), 74.9 (C-2′), 62.2 (C-5′), 43.7 (*CH*$_2C_5H_6$), 39.3, 34.7 ($CH_2$), 26.0, 25.2 (SiC(*CH*$_3)_3$), 19.7 ($CH_2$), 18.4, 17.8 (Si*C*$(CH_3)_3$), 12.2 ($CH_3$ base), −4.5, −4.8, −5.1, −5.7 (Si$(CH_3)_2$); HRMS : $C_{36}H_{56}N_4O_{10}SSi_2Na$ calcd. 815.3153, found 815.3151.

*4.3.2.9. [1-[2′,5′-Bis-O-tert-butyldiméthylsilyl-β-d-ribofuranosyl]-3-N-(methoxyisophtaloyl) thymine]-3′spiro-5″-(4″-amino-1″,2″-oxathiole-2″,2″-dioxide) (**17**).* TSAO-T (52 mg, $9.6 \times 10^{-2}$ mmol), $Na_2CO_3$ (74 mg, 0.70 mmol) and $Bu_4NBr$ (2.8 mg, $8.7 \times 10^{-3}$ mmol) were dissolved in a mixture of $CH_2Cl_2$ (3 mL) and water (6 mL). Isophtaloyle dichloride (23 mg, 0.11 mmol) was added to the mixture with vigorous stirring at 8 °C until disappearance of the starting material (mass spectrometry monitoring). After 15 min the reaction mixture was quenched with MeOH (2 mL, 0.049 mol). Vigorous stirring was continued at room temperature between 5 and 15 min. Water (10 mL) and $CH_2Cl_2$ (10 mL) were added; the organic phase was separated, washed with water (3 × 10 mL), dried ($Na_2SO_4$) and concentrated. The crude product was purified by HPLC to give **17** (28 mg, 42%). $R_f = 0.48$ (EtOAc/Cyclohexane : 5/5); Mp = 99–100 °C; $[\alpha]_D^{21}$: −26 (*c* 0.1, $CH_2Cl_2$); $^1H$ NMR ($CDCl_3$; 300 MHz) : δ 8.57 (s, 1H, H-1 $C_6H_4$), 8.35 (d, $J_{4,2}$ = 7.8 Hz, 1H, H-4 $C_6H_4$), 8.14 (d, $J_{2,3}$ = 7.8 Hz, 1H, H-2 $C_6H_4$), 7.63 (m, 1H, H-3 $C_6H_4$), 7.35 (s, 1H, H-6), 5.89 (d, $J_{1',2'}$ = 7.8 Hz, 1H, H-1′), 5.65 (s, 1H, H-3″), 5.51 (s, 2H, $NH_2$), 4.65 (d, 1H, H-2′), 4.37 (m, 1H, H-4′), 4.02 (dd, $J_{5'a,4'}$ = 2.6 Hz, $J_{5'a,5'b}$ = 12.4 Hz, 1H, H-5′a), 3.97 (s, 3H, $OCH_3$), 3.92 (dd, $J_{5'b,4'}$ = 2.7 Hz, 1H, H-5′b), 2.05 (s, 3H, $CH_3$ base), 0.99 (s, 9H, SiC$(CH_3)_3$), 0.89 (s, 9H, SiC$(CH_3)_3$), 0.23 (s, 3H, Si$(CH_3)_2$), 0.22 (s, 3H, Si$(CH_3)_2$), 0.09 (s, 3H, Si$(CH_3)_2$), −0.04 (s, 3H, Si$(CH_3)_2$); $^{13}C$ NMR ($CDCl_3$; 75 MHz) : δ 167.9 (*CO*$C_6H_4$), 165.6 (*CO*OMe), 162.5 (C-4), 150.4 (C-4″), 149.4 (C-2), 135.9 (C-6), 135.8–129.4 ($C_6H_4$), 111.9 (C-5), 93.1 (C-3″), 92.2 (C-3′), 87.6 (C-1′), 83.8 (C-4′), 74.6 (C-2′), 62.1 (C-5′), 52.5 (OMe), 26.0, 25.3 (SiC(*CH*$_3)_3$), 18.4, 17.9 (Si*C*$(CH_3)_3$), 12.3 ($CH_3$ base), −4.5, −4.9, −5.0, −5.7 (Si$(CH_3)_2$). HRMS : $C_{33}H_{49}N_3O_{11}SSi_2Na$ calcd. 774.2524, found 774.2532.

### *4.4. Biological methods*

#### *4.4.1. HIV*

A total number of 4 x $10^6$ CEM cells per milliliter were infected with HIV-1 ($III_B$) or HIV-2 (ROD) or HIV-1/E138K (a virus strain that was selected in the presence of TSAO-m$^3$T and that contained the E138K mutation in its RT) at ∼ 100 $CCID_{50}$ (50% cell culture infective dose) per milliliter of cell suspension. Then an amount of 100 μl of the infected cell suspension was transferred to microtiter plate wells and mixed with 100 μl of the appropriate dilutions of the test compounds. Syncytia formation was recorded microscopically in the HIV-infected cell cultures after 4 days. The 50% effective concentration ($EC_{50}$) of the test compounds was defined as the compound concentration required to inhibit virus-induced cytopathicity (CEM) by 50%. The 50% cytostatic/cytotoxic concentration ($CC_{50}$) was defined as the compound concentration required to inhibit CEM cell proliferation by 50%.

#### *4.4.2. HCV*

*4.4.2.1. Cell culture.* Huh-7 human hepatoma cells [37] were grown in Dulbecco's modified essential medium (Invitrogen) supplemented with 10% fetal bovine serum.

*4.4.2.2. HCV replication assay.* A modified assay based on a published method [38] was used. In this work, we performed a modified version containing a Luciferase reporter gene of the plasmid encoding JFH1 genome (genotype 2a), kindly provided by T. Wakita [39] (National Institute of Infectious Diseases, Tokyo, Japan).

Plasmid encoding the wild-type HCV genome was linearized at the 3′ end of the HCV cDNA with the restriction enzyme XbaI and treated with the Mung Bean Nuclease (New England Biolabs). *In vitro* transcripts were generated using the Megascript kit according to the manufacturer's protocol (Ambion). The *in vitro* reaction was set up and incubated at 37 °C for 4 h and transcripts were precipitated by addition of LiCl. Ten micrograms of RNA were delivered into Huh-7 cells by electroporation as described previously [40]. Huh-7 electroporated cells were seeded at a density of $2 \times 10^4$ cells/well in 96-well plates. Compounds were dissolved in DMSO, diluted in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum in a serial fashion to create an appropriate range of concentrations, and added to cells approximatively 24 h after plating. After 72 h exposure, the media was discarded from the assay plate and cell monolayers were lysed by addition of 100 μl of *Renilla* Luciferase Assay Lysis Buffer (Promega) with incubation at 20 °C for 30 min on an orbital shaker. Following incubation, luminescence was assessed as indicated by the manufacturer (Promega) on a CentroXS$^3$ LB960 Berthold. $EC_{50}$ and $CC_{50}$ values were determined using plots of luminescence versus log compound concentration.

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## Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.08.017.

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